Process for the Production of L-Amino Acids Using Strains of the Enterobacteriaceae Family

Field of the Invention

This invention relates to a process for the fermentative production of L-amino acids, particularly L-threonine, using strains of the Enterobacteriaceae family in which the open reading frame (ORF) having the designation yfiD and/or the pflB gene is/are enhanced.

Background of the Invention

L-amino acids such as L-threonine are used in human medicine, in the pharmaceutical industry, in the food industry and, very particularly, in animal nutrition. It is known that L-amino acids can be prepared by the fermentation of strains of Enterobacteriaceae, especially Escherichia coli (*E. coli*) and Serratia marcescens. As a result of the great importance of these amino acids, efforts are constantly made to improve production methods. Process improvements may relate to fermentation engineering measures, *e.g.*, methods of stirring and supplying oxygen, or to the composition of the nutrient media, *e.g.*, the sugar concentration present during fermentation. Alternatively, improvements may relate to the way in which product is purified, *e.g.*, ion-exchange chromatography, or to the intrinsic performance characteristics of the microorganism itself.

Methods of mutagenesis, selection and mutant choice are often used to improve the performance characteristics of microorganisms. In this way, strains are obtained that are resistant to antimetabolites such as the threonine analog α-amino-β-hydroxyvaleric acid (AHV) or that are auxotrophic for regulatorily important metabolites and which produce L-amino acids such as L-threonine. For some time now, methods of recombinant DNA engineering have also been used for improving L-amino acid-producing strains of the Enterobacteriaceae family. This often involves amplifying individual amino acid biosynthesis genes and testing the effect of this amplification on production. A summary of information relating to the cellular biology and molecular biology of Escherichia coli and Salmonella can be found in Neidhardt (ed.): Escherichia coli and Salmonella, Cellular and Molecular Biology, 2nd edition, ASM Press, Washington, D.C., USA,(1996).

Object of the Invention

The object of the present invention is to provide new measures for the improved fermentative production of L-amino acids and, in particular, L-threonine.

Summary of the Invention

The invention provides a process for the fermentative production of L-amino acids using microorganisms from the Enterobacteriaceae family in which at least the yfiD open reading frame (ORF) and/or the pflB gene, or nucleotide sequence(s) or alleles coding for the products thereof, is/are overexpressed.

In its first aspect, the invention is directed to a process for the production of an L-amino acid product by fermenting a recombinant microorganism from the Enterobacteriaceae family (preferably from the genus Escherichia, Erwinia, Providencia, or Serratia) in a fermentation medium. The recombinant microorganism produces the desired L-amino acid and is characterized by increased activity of the yfiD ORF product and/or the pflB gene product.

Increased activity may be due to the overexpression of the yfiD ORF or pflD gene endogenously present or due to the expression of another nucleotide sequence coding for the yfiD ORF product and/or the pflB gene product. One method for increasing the expression of polynucleotides is to increase copy number by at least 1. Increase in copy number can be achieved by integration of the gene or ORF into the chromosome of the microorganism or by means of an extra-chromosomally replicating vector. Alternatively, expression may be increased by mutating or replacing the promoter or ribosome binding site upstream of the yfiD ORF and/or the pflB gene. Preferably, recombinant engineering results in a concentration or activity of the yfiD gene product and/or of the pflB gene product (protein) that is increased by at least 10 %, relative to the activity or concentration of the gene product in the initial strain.

After allowing the desired amino acid to become enriched in either the fermentation medium or in the microorganism itself, it is isolated to produce the L-amino acid product. Amino acids that may be produced using this process include L-asparagine, L-serine, L-

glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan L-threonine, L-homoserine and L-arginine. The most preferred of these is L-threonine. It is also preferred that some or all of the constituents of the fermentation broth and/or biomass from the microorganism undergoing fermentation remain in the final amino acid product.

The process described above may be carried out using a microorganism in which, in addition to enhanced activity of the yfiD ORF product and/or the pflB gene product, at least one gene in a biosynthesis pathway of the L-amino acid being produced is also overexpressed. Examples of specific genes that may be overexpressed include:

a) the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase;
b) the pyc gene coding for pyruvate carboxylase;
c) the pps gene for phosphoenolpyruvate synthase;
d) the ppc gene coding for phosphoenolpyruvate carboxylase;
e) the genes pntA and pntB coding for transhydrogenase;
f) the rhtB gene imparting homoserine resistance;
g) the mqo gene coding for malate:quinone oxidoreductase;
h) the rhtC gene imparting threonine resistance;
i) the thrE gene coding for the threonine-export protein;
j) the gdhA gene coding for glutamate dehydrogenase;
k) the hns gene coding for the DNA binding protein HLP-II;
l) the pgm gene coding for phosphoglucomutase;
m) the fba gene coding for fructose biphosphate aldolase;
n) the ptsH gene coding for phosphohistidine protein hexose phosphotransferase;
o) the ptsI gene coding for enzyme I of the phosphotransferase system;
p) the crr gene coding for the glucose-specific IIA component;
q) the ptsG gene coding for the glucose-specific IIBC component;
r) the lrp gene coding for the regulator of the leucine regulon;
s) the csrA gene coding for the global regulator Csr;
t) the fadR gene coding for the regulator of the fad regulon;

u) the iclR gene coding for the regulator of central intermediary metabolism;

v) the mopB gene coding for the 10 kDa chaperon;

w) the ahpC gene coding for the small subunit of alkyl hydroperoxide reductase;

x) the ahpF gene coding for the large subunit of alkyl hydroperoxide reductase;

y) the cysK gene coding for cysteine synthase A;

z) the cysB gene coding for the regulator of the cys regulon;

aa) the cysJ gene coding for the flavoprotein of NADPH sulfite reductase;

bb) the cysI gene coding for the haemoprotein of NADPH sulfite reductase;

cc) the cysH gene coding for adenylyl sulfate reductase;

dd) the phoB gene coding for the positive regulator PhoB of the pho regulon;

ee) the phoR gene coding for the sensor protein of the pho regulon;

ff) the phoE gene coding for protein E of the outer cell membrane;

gg) the pykF gene coding for pyruvate kinase I, which is stimulated by fructose;

hh) the pfkB gene coding for 6-phosphofructokinase II;

ii) the malE gene coding for the periplasmic binding protein of maltose transport;

jj) the sodA gene coding for superoxide dismutase;

kk) the rseA gene coding for a membrane protein with anti-sigmaE activity;

ll) the rseC gene coding for a global regulator of the sigmaE factor;

mm) the sucA gene coding for the decarboxylase subunit of 2-ketoglutarate dehydrogenase;

nn) the sucB gene coding for the dihydrolipoyl transsuccinase E2 subunit of 2-ketoglutarate dehydrogenase;

oo) the sucC gene coding for the β-subunit of succinyl-CoA synthetase;

pp) the sucD gene coding for the α-subunit of succinyl-CoA synthetase;

qq) the adk gene coding for adenylate kinase;

rr) the hdeA gene coding for a periplasmic protein with chaperonin-type function;

ss) the hdeB gene coding for a periplasmic protein with chaperonin-type function;

tt) the icd gene coding for isocitrate dehydrogenase;

uu) the mglB gene coding for the periplasmic, galactose-binding transport protein;

vv) the lpd gene coding for dihydrolipoamide dehydrogenase;

ww) the aceE gene coding for the E1 component of the pyruvate-dehydrogenase complex;

xx) the aceF gene coding for the E2 component of the pyruvate-dehydrogenase complex;

yy) the pepB gene coding for aminopeptidase B;

zz) the aldH gene coding for aldehyde dehydrogenase,

aaa) the bfr gene coding for the iron-storage homoprotein;

bbb) the udp gene coding for uridine phosphorylase; and

ccc) the rseB gene coding for the regulator of sigmaE-factor activity.

Alternatively, a microorganism may be used in which, in addition to enhanced activity of the yfiD ORF product and/or the pflB gene product, the activity of the product of one or more additional genes is attenuated or eliminated or the expression of one or more additional genes or ORFs is diminished. Specific genes that may be attenuated by either being switched off or having their expression reduced, include:

a) the tdh gene coding for threonine dehydrogenase;

b) the mdh gene coding for malate dehydrogenase;

c) the open reading frame (ORF) yjfA;

d) the open reading frame (ORF) ytfP;

e) the pckA gene coding for phosphoenolpyruvate carboxykinase;

f) the poxB gene coding for pyruvate oxidase;

g) the aceA gene coding for isocitrate lyase;

h) the dgsA gene coding for the DgsA regulator of the phosphotransferase system;

i) the fruR gene coding for the fructose repressor;

j) the rpoS gene coding for the sigma38 factor;

k) the aspA gene coding for aspartate ammonium lyase; and

l) the aceB gene coding for malate synthase A.

In another aspect, the invention includes a microorganism from the Enterobacteriaceae family, in which the activity of the product of the yfiD ORF and/or the pflB gene is enhanced, *e.g.*, due to overexpression of the yfiD ORF or the pflB gene or due to the expression of other nucleotide sequences coding for the same products. Preferably, the microorganism is from the genus Escherichia and produces L-threonine.

Brief Description of the Figures:

Figure 1: Map of the vector pTrc99AyfiD.

Figure 2: Map of the vector pTrc99ApflB.

In both Figure 1 and Figure 2, length data are to be interpreted as approximate. The abbreviations and designations that are used have the following significance:

- Amp: ampicillin-resistance gene;
- lacI: gene for the repressor protein of the trc promoter;
- Ptrc: trc promoter region, IPTG-inducible;
- yfiD: coding region of the open reading frame yfiD;
- pflB: coding region of the pflB gene;
- 5S: 5S rRNA region;
- rrnBT: rRNA terminator region.

The abbreviations for the restriction enzymes have the following significance:

- HindIII: restriction endonuclease from Haemophilus influenze R_C;
- HpaI: restriction endonuclease from Haemophilus parainfluenzae;
- PauI: restriction endonuclease from Paracoccus alcaliphilus;
- XbaI: restriction endonuclease from Xanthomonas campestris.

Definitions

When L-amino acids or amino acids are mentioned herein, it will be understood that this means one or more amino acids, including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-threonine is particularly preferred.

The term "enhancement" in this context describes the increase in the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are encoded by the corresponding DNA. Enhancement may result from, for example: the number of copies of a gene or ORF being increased by at least one (1) copy; the use of a strong promoter; the use of a gene or allele that codes for a corresponding enzyme or protein with a high activity; and, optionally, by combining these measures.

The expression "open reading frame" (ORF) designates a segment of a nucleotide sequence that codes for, or can code for, a protein or, to be more exact, a polypeptide or ribonucleic acid, to which, according to the state of the art, no function can be assigned. After assignment of a function to the segment of the nucleotide sequence in question, one generally speaks of a gene.

The term "alleles" is generally understood to mean alternative forms of a given gene. The forms are distinguished by differences in the nucleotide sequence.

The expression "gene product" designates, in general, the protein encoded by a nucleotide sequence, *i.e.* an ORF, a gene or an allele, or the encoded ribonucleic acid.

By the measures of enhancement, in particular overexpression, the activity or concentration of the corresponding protein is generally increased by at least 10 %, 25 %, 50 %, 75 %, 100 %, 150 %, 200 %, 300 %, 400 % or 500 %, up to a maximum of 1000 % or 2000 %, relative to that of the wild-type protein or, to be more exact, the activity or concentration of the protein in the initial microorganism.

The expression "initial microorganism" or "parent strain" is understood to mean the microorganism in respect of which the measures according to the invention are carried out.

Detailed Description of the Invention

The present invention provides a process for the production of L-amino acids by fermentation of recombinant microorganisms of the Enterobacteriaceae family, characterized in that:

a) the microorganisms producing the desired L-amino-acid, in which the yfiD ORF and/or the pflB gene or nucleotide sequences or alleles coding for the gene products are enhanced, in particular overexpressed, are cultured in a medium under conditions in which the desired L-amino acid in the medium or in the cells is enriched, and

b) the desired L-amino acid is isolated, whereby optionally constituents of the fermentation broth and/or the biomass in its entirety or in portions (> 0 to 100 %) remain in the isolated product or are completely removed.

The microorganisms, in particular recombinant microorganisms, which are provided by the present invention, are able to produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, in appropriate circumstances starch, in appropriate circumstances cellulose, or from glycerin and ethanol. Such microorganisms are representatives of the Enterobacteriaceae family, preferably selected from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are especially preferred. In the case of the genus Escherichia, the most preferred species is Escherichia coli, and in the case of the genus Serratia, the most preferred species is Serratia marcescens.

In general, recombinant microorganisms are generated by transformation, transduction or conjugation with a vector carrying the desired gene. Suitable strains of the genus Escherichia, in particular of the species Escherichia coli, which in particular, produce L-threonine, are, for example:

- Escherichia coli H4581, (EP 0 301 572);
- Escherichia coli KY10935, (*Biosci. Biotechnol. Biochem. 61*(11):1877-1882 (1997);
- Escherichia coli VNIIgenetika MG442, (US-A-4278,765);
- Escherichia coli VNIIgenetika M1, (US-A-4,321,325);

- Escherichia coli VNIIgenetika 472T23, (US-A-5,631,157);
- Escherichia coli BKIIM B-3996, (US-A-5,175,107);
- Escherichia coli kat 13, (WO 98/04715); and
- Escherichia coli KCCM-10132 (WO 00/09660).

Suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens, are, for example:

- Serratia marcescens HNr21, (*Appl. and Envir. Microbiol. 38*(6):1045-1051 (1979));
- Serratia marcescens TLr156 (*Gene 57*(2-3):151-158 (1987)); and
- Serratia marcescens T-2000, (*Appl. Biochem. Biotechnol. 37*(3):255-265 (1992)).

L-threonine-producing strains from the Enterobacteriaceae family preferably possess, *inter alia*, one or more of the genetic or phenotypic features selected from the group comprising: resistance to α-amino-β-hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α-methylserine, resistance to diaminosuccinic acid, resistance to α-aminobutyric acid, resistance to borrelidin, resistance to cyclopentanecarboxylic acid, resistance to rifampicin, resistance to valine analogues such as, for example, valine hydroxamate, resistance to purine analogues such as, for example, 6-dimethylaminopurine, need for L-methionine, in appropriate circumstances partial and compensable need for L-isoleucine, need for meso-diaminopimelic acid, auxotrophy with respect to threonine-containing dipeptides, resistance to L-threonine, resistance to threonine raffinate, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, in appropriate circumstances the ability to utilize sucrose, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feedback-resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feedback-resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenolpyruvate carboxylase, optionally of the feedback-resistant form, enhancement of phosphoenolpyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene

product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of the formation of acetic acid.

After enhancement, in particular overexpression, of the open reading frame yfiD and/or of the pflB gene or nucleotide sequence(s) or alleles coding for the corresponding gene products, microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner. The nucleotide sequences of the genes or open reading frames (ORFs) of Escherichia coli can be found in the genome sequence of Escherichia coli published by Blattner *et al.* (*Science 277*:1453–1462 (1997)). The open reading frame yfiD and the protein coded by this ORF are described, *inter alia*, by the following data:

Designation: open reading frame;
Function: putative formate acetyl transferase;
Description: the open reading frame yfiD codes for a 14.3 kDa protein, the isoelectric point is situated at 5.1; localized chromosomally, it is situated, for example in the case of Escherichia coli K12 MG1655, in the intergenic region of the open reading frame yfiK, coding for a putative L-aspartate oxidase, and the ung gene, coding for uracil DNA glycosylase
Reference: Blankenhorn *et al.*; *J. Bacteriol. 181*(7):2209-2216 (1999);
Fountoulakis *et al.*; *Electrophoresis 20*(11): 2181-2195 (1999);
Kirkpatrick *et al.*; *J. Bacteriol. 183*(21):6466-6477 (2001);
Wyborn *et al.*; *Microbiol. 148*:1015-1026 (2002).
Accession No.: AE000344

The pflB gene and the protein coded by this gene are described, *inter alia*, by the following data:

Designation: formate acetyl transferase I, pyruvate formate lyase I
EC No.: 2.3.1.54
Reference: Rodel *et al.*; *Eur. J. Biochem. 177*(1):153-158 (1988);
Wagner *et al.*; *Proc. Nat'l Acad. Sci. USA 89*(3): 996-1000 (1992).

Accession No.: AE000192

Alternative gene name: pfl

The pyruvate formate lyase from Salmonella typhimurium is described, inter alia, in Wong *et al.*, *J. Bacteriol. 171*(9):4900-4905 (1989).

The nucleic-acid sequences can be obtained from the databases of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), from the Nucleotide Sequence Database of the European Molecular Biology Laboratory (EMBL, Heidelberg, Germany, and Cambridge, UK) or from the DNA Data Bank of Japan (DDBJ, Mishima, Japan). For the sake of better clarity, the known sequence relating to the yfiD ORF is represented herein as SEQ ID NO:3. The protein coded by this reading frame is represented herein as SEQ ID NO:4.

The sequence specified in the sequence listing can be used in accordance with the invention. Use may also be made of alleles of the genes or open reading frames that result from the degeneracy of the genetic code or by virtue of functionally neutral sense mutations. The use of endogenous genes or of endogenous open reading frames is preferred. The expression "endogenous genes" or "endogenous nucleotide sequences" is understood to mean the genes or open reading frames or alleles or, to be more exact, nucleotide sequences, that are present in the population of a species.

The alleles that contain functionally neutral sense mutations include, inter alia, those which result in at least one (1) conservative amino-acid exchange in the protein coded by them. In the case of the aromatic amino acids, conservative exchanges occur when phenylalanine, tryptophan and tyrosine are exchanged for one another. In the case of the hydrophobic amino acids, conservative exchanges occur if leucine, isoleucine and valine are exchanged for one another. In the case of the polar amino acids, conservative exchanges occur if glutamine and asparagine are exchanged for one another. In the case of the basic amino acids, conservative exchanges occur if arginine, lysine and histidine are exchanged for one another. In the case of the acidic amino acids, conservative exchanges occur if aspartic acid and glutamic acid are exchanged for one another. In the case of the amino acids

containing hydroxyl groups, conservative exchanges occur if serine and threonine are exchanged for one another.

Similarly, nucleotide sequences can be used which code for variants of the stated proteins which additionally contain at the N-terminus or C-terminus a lengthening or shortening of at least one (1) amino acid. This lengthening or shortening amounts to not more than 50, 40, 30, 20, 10, 5, 3 or 2 amino acids or amino-acid residues.

Suitable alleles also include those which code for proteins in which at least one (1) amino acid is inserted or deleted. The maximum number of such changes, which are designated as indels, may concern 2, 3, 5, 10, 20 but in no case more than 30 amino acids. The suitable alleles include, furthermore, those which can be obtained by hybridization, in particular under stringent conditions using SEQ ID NO:3 or SEQ ID NO:7 or parts thereof, particularly the coding regions or the sequences complementary thereto. Instructions on the identification of DNA sequences by means of hybridization can be found by a person skilled in the art, *inter alia*, in the manual entitled "The DIG System Users Guide for Filter Hybridization" produced by Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl *et al.* (*Internat'l J. Systematic Bacteriol. 41*:255-260 (1991)). The hybridization takes place under stringent conditions - *i.e.*, only hybrids are formed in which the probe and the target sequence, *i.e.* the polynucleotides treated with the probe, are at least 70 % identical. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the composition of the buffer, the temperature, and the salt concentration. The hybridization reaction is generally carried out with relatively low stringency in comparison with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

For the hybridization reaction, a buffer corresponding to 5x SSC buffer at a temperature of about 50°C - 68°C can be employed. Under these conditions, probes can hybridize with polynucleotides that exhibit less than 70 % identity to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be attained, for example, by lowering the salt concentration to 2x SSC and optionally subsequently to 0.5x SSC (The DIG System User's Guide for Filter

Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), at a temperature of about 50°C – 68°C, about 52°C – 68°C, about 54°C – 68°C, about 56°C – 68°C, about 58°C – 68°C, about 60°C – 68°C, about 62°C – 68°C, about 64°C – 68°C or about 66°C – 68°C. Optionally, he salt concentration may be lowered to a concentration corresponding to 0.2x SSC or 0.1x SSC. By stepwise increase of the hybridization temperature in steps of about 1 – 2 °C from 50 °C to 68 °C, polynucleotide fragments can be isolated which, for example, possess at least 70 % or at least 80 % or at least 90 % to 95 % or at least 96 % to 99 % identity to the sequence of the probe employed. Further instructions on hybridization are commercially obtainable in the form of so-called kits (*e.g.*, DIG Easy Hyb produced by Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

To enhance activity, the expression of the genes or open reading frames or alleles can be increased, or the catalytic or regulatory properties (activity) of the proteins can be enhanced. Both measures may optionally be combined.

Overexpression may be accomplished by increasing the number of copies of the corresponding genes or open reading frames, or by mutating the promoter and regulation region or the ribosome binding site, which is located upstream of the structural gene. Expression cassettes which are incorporated upstream of the structural gene act in like manner. Expression can also be increased using inducible promoters or by prolonging the lifespan of mRNA. By preventing the degradation of the enzyme protein, enzyme activity is likewise enhanced. The genes or gene constructs may either be present in extra-chromosomally replicating plasmids with a different number of copies or may be integrated within the chromosome and amplified.

Alternatively overexpression can be obtained by changing the composition of the media and by culture management. Instructions on this can be found, *inter alia*, in Chang, *et al.* (*J. Bacteriol. 134*:1141-1156 (1978)), in Hartley, *et al.* (*Gene 13*:347-353 (1981)), in Amann, *et al.* (*Gene 40*:183-190 (1985)), in de Broer, *et al.* (*Proc. Nat'l Acad. Sci. USA 80*: 21-25 (1983)), in LaVallie *et al.* (*BIO/TECHNOLOGY 11*:187-193 (1993)), in WO98/04715, in Llosa, *et al.* (*Plasmid 26*:222-224 (1991)), in Quandt, *et al.*, (*Gene 80*: 61-

169 (1989)), in Hamilton, *et al.* (*J. Bacteriol. 171*:4617-4622 (1989)), in Jensen, *et al.*, (*Biotech. Bioeng. 58*:191-195 (1998)) and in textbooks on genetics and molecular biology.

Use may be made of plasmid vectors capable of replicating in Enterobacteriaceae, such as cloning vectors derived from pACYC184 (Bartolomé, *et al.*, *Gene 102*:75-78 (1991)), pTrc99A (*Amann et al.*; *Gene 69*:301-315 (1988)) or pSC101 derivatives (Vocke, *et al.*, *Proc. Nat'l Acad. Sci. USA 80*(21):6557-6561 (1983)). A strain transformed with a plasmid vector can be employed in a process according to the invention, in which case the plasmid vector carries at least the yfiD ORF and/or the pflB gene or nucleotide sequences or alleles coding for the gene products thereof.

The term "transformation refers to the uptake of a nucleic acid by a host (microorganism). It is likewise possible to transfer mutations that relate to the expression of the respective genes or open reading frames into various strains by sequence exchange (Hamilton *et al.*, *J. Bacteriol. 171*:4617–4622 (1989)), conjugation or transduction. Detailed explanations relating to the terms of genetics and molecular biology can be found in textbooks on genetics and molecular biology, such as the textbook by Birge (Bacterial and Bacteriophage Genetics, 4th ed., Springer Verlag, New York (USA), 2000), the textbook by Berg, *et al.* (Biochemistry, 5th ed., Freeman and Company, New York (USA), 2002), or the textbook by Sambrook, *et al.* (Molecular Cloning, A Laboratory Manual, (3-volume set), Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001).

The production of L-amino acids may also be improved by enhancing one or more enzymes of the known threonine-biosynthesis pathway, enzymes of anaplerotic metabolism, enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate, enzymes of glycolysis, PTS enzymes, or enzymes of sulfur metabolism. The use of endogenous genes is generally preferred. Examples of genes that may be enhanced, preferably by being overexpressed, include:

- the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765);

- the pyc gene of Corynebacterium glutamicum coding for pyruvate carboxylase (WO 99/18228);
- the pps gene coding for phosphoenolpyruvate synthase (*Mol. Gen. Genet. 231*(2):332-336 (1992));
- the ppc gene coding for phosphoenolpyruvate carboxylase (*Gene 31*:279-283 (1984));
- the genes pntA and pntB coding for transhydrogenase (*Eur. J. Biochem. 158*:647-653 (1986));
- the gene rhtB imparting homoserine resistance (EP-A-0 994 190);
- the mqo gene coding for malate:quinine oxidoreductase (WO 02/06459);
- the rhtC gene imparting threonine resistance (EP-A-1 013 765);
- thrE gene of Corynebacterium glutamicum coding for the threonine-export protein (WO 01/92545);
- the gdhA gene coding for glutamate dehydrogenase (*Nucl. Ac. Res. 11*:5257-5266 (1983); *Gene 23*:199-209 (1983));
- the hns gene coding for the DNA binding protein HLP-II (WO 03/004671);
- the pgm gene coding for phosphoglucomutase (WO 03/004598);
- the fba gene coding for fructose biphosphate aldolase (WO 03/004664);
- the ptsH gene of the ptsHIcrr operon coding for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (WO 03/004674);
- the ptsI gene of the ptsHIcrr operon coding for enzyme I of the phosphotransferase system PTS (WO 03/004674);
- the crr gene of the ptsHIcrr operon coding for the glucose-specific IIA component of the phosphotransferase system PTS (WO 03/004674);
- the ptsG gene coding for the glucose-specific IIBC component (WO 03/004670);
- the lrp gene coding for the regulator of the leucine regulon (WO 03/004665);
- the csrA gene coding for the global regulator Csr (*J. Bacteriol. 175*:4744-4755 (1993));
- the fadR gene coding for the regulator of the fad regulon (*Nucl. Ac. Res. 16*:7995-8009 (1988));
- the iclR gene coding for the regulator of central intermediary metabolism (*J. Bacteriol. 172:* 2642-2649 (1990));

- the mopB gene coding for the 10 kDa chaperon (WO 03/004669), which is also known under the designation "groES;"
- the ahpC gene of the ahpCF operon coding for the small subunit of alkyl hydroperoxide reductase (WO 03/004663);
- the ahpF gene of the ahpCF operon coding for the large subunit of alkyl hydroperoxide reductase (WO 03/004663);
- the cysK gene coding for cysteine synthase A (WO 03/006666);
- the cysB gene coding for the regulator of the cys regulon (WO 03/006666);
- the cysJ gene of the cysJIH operon coding for the flavoprotein of NADPH sulfite reductase (WO 03/006666);
- the cysI gene of the cysJIH operon coding for the haemoprotein of NADPH sulfite reductase (WO 03/006666);
- the cysH gene of the cysJIH operon coding for adenylyl sulfate reductase (WO 03/006666);
- the phoB gene of the phoBR operon coding for the positive regulator PhoB of the pho regulon (WO 03/008606);
- the phoR gene of the phoBR operon coding for the sensor protein of the pho regulon (WO 03/008606);
- the phoE gene coding for protein E of the outer cell membrane (WO 03/008608);
- the pykF gene coding for pyruvate kinase I which is stimulated by fructose (WO 03/008609);
- the pfkB gene coding for 6-phosphofructokinase II (WO 03/008610);
- the malE gene coding for the periplasmic binding protein of maltose transport (WO 03/008605);
- the sodA gene coding for superoxide dismutase (WO 03/008613);
- the rseA gene of the rseABC operon coding for a membrane protein with anti-sigmaE activity (WO 03/008612);
- the rseC gene of the rseABC operon coding for a global regulator of the sigmaE factors (WO 03/008612);
- the sucA gene of the sucABCD operon coding for the decarboxylase subunit of 2-ketoglutarate dehydrogenase (WO 03/008614);

- the sucB gene of the sucABCD operon coding for the dihydrolipoyl transsuccinase E2 subunit of 2-ketoglutarate dehydrogenase (WO 03/008614);
- the sucC gene of the suc ABCD operon coding for the β-subunit of succinyl-CoA synthetase (WO 03/008615);
- the sucD gene of the sucABCD operon coding for the α-subunit of succinyl-CoA synthetase (WO 03/008615);
- the adk gene coding for adenylate kinase (*Nucl. Ac. Res. 13*(19):7139-7151 (1985));
- the hdeA gene coding for a periplasmic protein with chaperonin-type function (*J. Bacteriol. 175*(23):7747-7748 (1993));
- the hdeB gene coding for a periplasmic protein with chaperonin-type function (*J. Bacteriol. 175*(23):7747-7748 (1993));
- the icd gene coding for isocitrate dehydrogenase (*J. Biol. Chem. 262*(22):10422-10425 (1987));
- the mglB gene coding for the periplasmic, galactose-binding transport protein (*Mol. Gen. Genet. 229*(3):453-459 (1991));
- the lpd gene coding for dihydrolipoamide dehydrogenase (*Eur. J. Biochem. 135*(3):519-527 (1983));
- the aceE gene coding for the E1 component of the pyruvate-dehydrogenase complex (*Eur. J. Biochem. 133*(1):155-162 (1983));
- the aceF gene coding for the E2 component of the pyruvate-dehydrogenase complex (*Eur. J. Biochem. 133*(3):481-489 (1983));
- the pepB gene coding for aminopeptidase B (*J. Fermentation Bioeng. 82*:392-397 (1996));
- the aldH gene coding for aldehyde dehydrogenase (E.C. 1.2.1.3) (*Gene 99*(1):15-23 (1991));
- the bfr gene coding for the iron-storage homoprotein (bacterioferritin) (*J. Bacteriol. 171*(7):3940-3947 (1989));
- the udp gene coding for uridine phosphorylase (*Nucl. Ac. Res. 17*(16): 6741 (1989)) and
- the rseB gene coding for the regulator of sigmaE-factor activity (*Mol. Microbiol. 24*(2): 355-371 (1997)) .

It may also be advantageous, in addition to the enhancement of the yfiD ORF and/or of the pflB gene, to attenuate (in particular to eliminate or to diminish the expression of) one or more of the following:

- the tdh gene coding for threonine dehydrogenase (*J. Bacteriol. 169*:4716-4721 (1987));
- the mdh gene coding for malate dehydrogenase (E.C. 1.1.1.37) (*Arch. Microbiol. 149*:36-42 (1987));
- the gene product of the open reading frame (ORF) yjfA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) WO 02/29080);
- the gene product of the open reading frame (ORF) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)), WO 02/29080);
- the pckA gene coding for the enzyme phosphoenolpyruvate carboxykinase (WO 02/29080);
- the poxB gene coding for pyruvate oxidase (WO 02/36797);
- the aceA gene coding for the enzyme isocitrate lyase (WO 02/081722);
- the dgsA gene coding for the DgsA regulator of the phosphotransferase system (WO 02/081721), which is also known under the designation "mlc gene;"
- the fruR gene coding for the fructose repressor (WO 02/081698), which is also known as the "cra gene;"
- the rpoS gene coding for the sigma^{38} factor (WO 01/05939), which is also known as the "katF gene;"
- the aspA gene coding for aspartate ammonium lyase (WO 03/008603); and
- the aceB gene coding for malate synthase A (WO 03/008604).

The term "attenuation" in this context describes the diminution or elimination of the intracellular activity or concentration of one or more enzymes or proteins in a microorganism that is/are encoded by the corresponding DNA. This may be accomplished, for example, through the use of a weak promoter or a gene or allele that codes for a corresponding enzyme or protein with a low activity or that inactivates the corresponding enzyme or protein or the open reading frame or the gene, and by optionally combining these measures. By the measures of attenuation, the activity or concentration of the corresponding

protein is generally lowered to 0 to 75 %, 0 to 50 %, 0 to 25 %, 0 to 10 % or 0 to 5 % of the activity or concentration of the wild-type protein or, to be more exact, of the activity or concentration of the protein in the initial microorganism.

The production of L-amino acids in microorganisms with enhanced activity of the yfiD ORF product and/or the pflB gene product, may also benefit from the elimination of one or more side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms," in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982). The microorganisms produced in accordance with the invention can be cultured in the batch process, in the fed-batch process or in the repeated fed-batch process. A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) and in the textbook by Storhas (Bioreaktoren and periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium that is to be used has to satisfy the demands of the respective strains in a suitable manner. Descriptions of culture media of various microorganisms are contained in the manual entitled Manual of Methods for General Bacteriology produced by The American Society for Bacteriology (Washington D.C., USA, 1981). For a carbon source, use may be made of sugar and carbohydrates such as glucose, sucrose, lactose, fructose, maltose, molasses, starch and, in appropriate circumstances, cellulose, oils and fats such as, for example, soybean oil, sunflower oil, peanut oil and copra oil, fatty acids such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols such as, for example, glycerin and ethanol, and organic acids such as, for example, acetic acid. These substances may be used individually or in the form of a mixture.

For a nitrogen source, use may be made of organic nitrogenous compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or in the form of a mixture.

For a phosphorus source, use may be made of phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts. The culture medium must also contain salts of metals, such as magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth-regulating substances such as amino acids and vitamins may be employed in addition to the aforementioned substances. Suitable precursors may, moreover, be added to the culture medium. The stated feed materials may be added to the culture in the form of a single batch or may be fed in during the cultivation in suitable manner.

The fermentation is generally carried out at a pH value from 5.5 to 9.0, in particular 6.0 to 8.0. For the purpose of controlling the pH of the culture, basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammoniacal liquor or acidic compounds such as phosphoric acid or sulfuric acid are employed in suitable manner. For the purpose of controlling the evolution of foam, anti-foaming agents such as, for example, fatty-acid polyglycol esters may be employed. For the purpose of maintaining the stability of plasmids, suitable substances acting selectively, for example antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as air for example, are introduced into the culture. The temperature of the culture is normally around 25 °C to 45 °C and preferably around 30 °C to 40 °C.

The culture is carried on until such time as a maximum of L-amino acids, preferably L-threonine, has formed. This objective is normally attained within 10 hours to 160 hours. The analysis of L-amino acids can be undertaken by anion-exchange chromatography with subsequent ninhydrin derivation, as described in Spackman, *et al.* (*Anal. Chem. 30*:1190-1206 (1958)), or it can be undertaken by reversed phase HPLC, as described in Lindroth, *et al.* (*Anal. Chem. 51*:1167-1174 (1979)).

The process according to the invention may be used for the fermentative production of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

The present invention may be further understood based upon the following non-limiting examples.

Examples

Minimal media (M9) and complete media (LB) that are used for Escherichia coli are described by J.H. Miller (A short course in bacterial genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and also all techniques relating to restriction, ligation, Klenow treatment and alkaline phosphatase treatment are carried out in accordance with Sambrook *et al.* (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). The transformation of Escherichia coli is carried out, unless described otherwise, in accordance with Chung, *et al.* (*Proc. Nat'l Acad. Sci. USA 86*:2172-2175 (1989)). The incubation temperature in the course of the production of strains and transformants is 37 °C.

Example 1

1.1 Construction of the expression plasmid pTrc99AyfiD

The open reading frame yfiD from E. coli K12 is amplified by using the polymerase chain reaction (PCR) and also synthetic oligonucleotides. Starting from the nucleotide sequence of the open reading frame yfiD in E. coli K12 MG1655 (Accession Number AE000344, Blattner *et al.* (*Science 277*:1453–1474 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The primers contain sequences for restriction enzymes, which are marked by underlining in the nucleotide sequence represented below. The primer yfiD1 contains the restriction site for XbaI; the primer yfiD2 contains the restriction site for HindIII.

yfiD1:

5' – GAACAAATCTAGAAATTAAGCCGGGGAGGC -3' (SEQ ID NO:1)

yfiD2:

5' – GCTACTTAAGCTTTACAGGCTTTC – 3' (SEQ ID NO:2)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated in accordance with the manufacturer's directions using "Qiagen Genomic-tips 100/G"

(QIAGEN, Hilden, Germany). A DNA fragment with a size of about 431 bp can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with Vent-DNA-Polymerase (New England Biolabs, Frankfurt, Germany) (SEQ ID NO:3).

The PCR product is restricted with the restriction enzymes HindIII and XbaI and examined in a 0.8 % agarose gel after being cleaned up (Purification Kit, QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI, and ligated with the restricted yfiD fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch, and plasmid-bearing cells are selected on LB agar to which 50 µg/ml ampicillin have been added. The successful cloning can be demonstrated after the isolation of plasmid DNA by control cleavage with the enzymes HindIII/XbaI and HpaI. The plasmid is designated as pTrc99AyfiD (Figure 1).

1.2 Construction of the expression plasmid pTrc99ApflB

The pflB gene from E. coli K12 is amplified by using the polymerase chain reaction (PCR) and also synthetic oligonucleotides. Starting from the nucleotide sequence of the pflB gene in E. coli K12 MG1655 (Accession Number AE000192, Blattner *et al.* (*Science 277*:1453–1474 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The primers contain sequences for restriction enzymes, which are marked by underlining in the nucleotide sequence represented below. The primer pflB1 contains the restriction site for XbaI; the primer pflB2 contains the restriction site for HindIII.

pflB1:

5' – CCACTCTAGAAGGTAGGTGTTACATGTC -3' (SEQ ID NO:5)

pflB2:

5' – CGATTTCAGTCAAAGCTTATTACATAG – 3' (SEQ ID NO:6).

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated in accordance with the manufacturer's directions using "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment with a size of about 2325 bp can be

amplified with the specific primers under standard PCR conditions (Innis *et al.* (1990) PCR Protocols. A guide to methods and applications, Academic Press) with Vent-DNA-Polymerase (New England Biolabs, Frankfurt, Germany) (SEQ ID No. 7).

The PCR product is restricted with the restriction enzymes HindIII and XbaI and examined in a 0.8 % agarose gel after being cleaned up (Purification Kit, QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI, and ligated with the restricted pflB fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch, and plasmid-bearing cells are selected on LB agar to which 50 µg/ml ampicillin have been added. The successful cloning can be demonstrated after the isolation of plasmid DNA by control cleavage with the enzymes HindIII/XbaI and PauI. The plasmid is designated as pTrc99ApflB (Figure 2).

Example 2

2.1 Production of L-threonine with the strain MG442/pTrc99AyfiD

The L-threonine-producing E. coli strain MG442 is described in patent specification US-A-4,278,765 and deposited at the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia) as CMIM B-1628. The strain MG442 is transformed with the expression plasmid pTrc99AyfiD described in Example 1.1 and with the vector pTrc99A, and plasmid-bearing cells are selected on LB agar with 50 µg/ml ampicillin. In this way, the strains MG442/pTrc99AyfiD and MG442/pTrc99A arise. Selected single colonies are subsequently multiplied further on minimal medium having the following composition: 3.5 g/l $Na_2HPO_4*2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4*7H_2O$, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is examined in batch cultures of 10 ml, which are contained in 100 ml Erlenmeyer flasks. To this end, 10 ml of preculture medium having the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose, 50 mg/l ampicillin, are inoculated and incubated for 16 hours at 37°C and at 180 rpm in an ESR incubator manufactured by Kühner AG (Birsfelden, Switzerland). 250 µl at a time of this preculture are inoculated into 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4*7H_2O$, 0.03 g/l $FeSO_4*7H_2O$, 0.018 g/l

$MnSO_4*1H_2O$, 30 g/l $CaCO_3$, 20 g/l glucose, 50 mg/l ampicillin) and incubated for 48 hours at 37°C. The formation of L-threonine by the initial strain MG442 is examined in the same way, there being, however, no addition of ampicillin to the medium. After the incubation, the optical density (OD) of the culture suspension is determined at a measuring wavelength of 660 nm with an LP2W photometer manufactured by Dr. Lange (Düsseldorf, Germany). Subsequently the concentration of L-threonine which has formed is determined in the sterile-filtered culture supernatant with an amino-acid analyzer manufactured by Eppendorf-BioTronik (Hamburg, Germany), by ion-exchange chromatography and post-column reaction with detection of ninhydrin. The result of the experiment is presented in Table 1.

Table 1

Strain	OD (660 nm)	L-threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AyfiD	5.5	2.5

2.2 Production of L-threonine with the strain MG442/pTrc99ApflB

The L-threonine-producing E. coli strain MG442 is described in patent specification US 4,278,765 and deposited at the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia) as CMIM B-1628. The strain MG442 is transformed with the expression plasmid pTrc99ApflB described in Example 1.2 and with the vector pTrc99A, and plasmid-bearing cells are selected on LB agar with 50 µg/ml ampicillin. In this way, the strains MG442/pTrc99ApflB and MG442/pTrc99A arise. Selected single colonies are subsequently multiplied further on minimal medium having the following composition: 3.5 g/l $Na_2HPO_4*2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4*7H_2O$, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is examined in batch cultures of 10 ml, which are contained in 100 ml Erlenmeyer flasks. To this end, 10 ml of preculture medium having the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose, 50

mg/l ampicillin, are inoculated and incubated for 16 hours at 37°C and 180 rpm in an ESR incubator manufactured by Kühner AG (Birsfelden, Switzerland). 250 µl at a time of this preculture are inoculated into 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4*7H_2O$, 0.03 g/l $FeSO_4*7H_2O$, 0.018 g/l $MnSO_4*1H_2O$, 30 g/l $CaCO_3$, 20 g/l glucose, 50 mg/l ampicillin) and incubated for 48 hours at 37°C. With a view to complete induction of the expression of the pflB gene, 100 mg/l isopropyl-β-D-thiogalactopyranoside (IPTG) are added in parallel batches. The formation of L-threonine by the initial strain MG442 is examined in the same way, there being, however, no addition of ampicillin to the medium. After the incubation, the optical density (OD) of the culture suspension is determined at a measuring wavelength of 660 nm with an LP2W photometer manufactured by Dr. Lange (Düsseldorf, Germany). Subsequently the concentration of L-threonine which has formed is determined in the sterile-filtered culture supernatant with an amino-acid analyzer manufactured by Eppendorf-BioTronik (Hamburg, Germany), by ion-exchange chromatography and post-column reaction with detection of ninhydrin. The result of the experiment is presented in Table 2.

Table 2

Strain	Additives	OD (660 nm)	L-threonine g/l
MG442	-	5.6	1.4
MG442/pTrc99A	-	3.8	1.3
MG442/pTrc99ApflB	-	5.6	1.9
MG442/pTrc99ApflB	IPTG	5.2	2.2

All references cited herein are fully incorporated by reference. Having now fully described the invention, it will be understood by those of skill in the art that the invention

may be performed within a wide and equivalent range of conditions, parameters and the like, without affecting the spirit or scope of the invention or any embodiment thereof.